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Note

Detection of *Aeromonas hydrophila* by polymerase chain reaction

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ABSTRACT

Nine isolates of *A. hydrophila* isolated from fish and water samples were selected for the present study. The detection of *A. hydrophila* was done by amplification of the *lip* gene through known primer sequences at a modified annealing temperature of 65^{0}C. A product size of 760 bp was found to be specific for the detection of *A. hydrophila* by PCR. No amplification product of similar size was obtained in *Salmonella typhimurium* and *Edwardseilla tarda*. This method can be used for the rapid and specific identification of *A.hydrophila* from aquatic environment.

The aquatic environment is considered to be the principal reservoir of *Aeromonas* spp. (Wadstrom and Ljungh, 1991). In India, the organism has been isolated from variety of foods including fish, meat, milk, eggs, tortoise and snails (Agarwal, 1997). Pathak *et al.* (1988) have reported fish as the chief source of this microorganism in India. *A. hydrophila* is considered to be the principal cause of bacterial hemorrhagic septicemia in fresh water fish and has been reported in association with various ulcerative syndrome and red spot disease. These infections can cause high mortalities in fish hatcheries and in natural waters.

The genus *Aeromonas* includes two well-separated groups of organisms, a psychrophilic, non-motile group and a mesophilic motile group. The latter includes three species viz., *A. hydrophila*, *A. caviae* and *A. sobria* (Popoff *et al.*, 1981). DNA homology studies have revealed that there are at least 14 hybridization groups within these species (Joseph and Carnahan, 1994). In food and environmental samples, they might be present in lower concentrations and are accompanied by large numbers of competing micro-flora. The conventional microbiological procedure and biochemical methods for isolating and identifying bacterial pathogens are rather labour intensive and time-consuming process. To overcome these problems, some commercial identification systems such as AP1 20E, AP1 20 NE and Microbact 24E have been developed and evaluated to identify *Aeromonas* spp. (Ogden *et al.*, 1994). However, they are not fully adequate for identification upto species level (Khan and Cerniglia, 1997).

Lately, emphasis has also been placed on the development of biotechnological and immunological methods for the detection of mesophilic

aeromonads. Polymerase chain reaction have been developed for detection of *Aeromonas* spp. from water samples and food products (Dorsch *et al.*, 1994 and Cascon *et al.*, 1996). Similarly DNA/RNA probes are also in use for this purpose (Dorsch *et al.*, 1994 and Ludwig *et al.*, 1994).

In the present study *A. hydrophila* was isolated from aquatic environment and its detection was attempted by polymerase chain reaction. The specificity of this detection test was also checked with other bacterial pathogens of the family Enterobacteriaceae. The primers selected in this study for the screening of *Aeromonas* spp. were designed on the basis of the published nucleotide sequence of *lip* gene (Cascon *et al.*, 1996).

Isolation of Aeromonas hydrophila

Samples of water were collected from aquarium and fish ponds of the National Bureau of Fish Genetic Resources (NBFGR), Lucknow. Water samples were also collected from ponds and a canal located in Telibagh area of Lucknow. Altogether 13 water samples and 7 fish samples were used for the screening of *A. hydrophila*. Species identification of *Aeromonas* was done by following Aero-key as proposed by Carnahan *et al.* (1991) with modifications suggested by Agarwal (1997). The isolates of *A. hydrophila*, reference strains of *Salmonella typhimurium* and *Edwardseilla tarda* were used for this study.

Isolation of genomic DNA

The genomic DNA was isolated as per the protocol described by Hiney *et al.* (1992). A single colony was inoculated in 10 ml of Nutrient broth (NB) and grown at 29°C overnight. Culture was centrifuged at 5000 rpm for 10 minutes. Four hundred microlitre of solution I (50mM Tris.HCl pH-8.0, 50mM EDTA pH-8.0, 25% sucrose, 1mg lysozyme), was added to the washed cell pellet and gently mixed and incubated at 37° C for 15 minutes. Thereafter 400ml of solution II (10mM Tris.HCl pH 8.0, 5mM EDTA pH-8.0, 1% SDS, 40µg Proteinase K) was added to the cells and incubated at 55° C for three hours. During the incubation period the cells were mixed gently at periodic intervals. Equal amount of phenol: chloroform mixture was added to the cell lysate and mixed by the inversion of tube 2 to 3 times. The suspension was centrifuged at 6000 rpm for 10 minutes. The aqueous layer from the top was removed carefully to avoid any protein debris and transferred to a fresh microfuge tube. Double amount of chilled ethanol was added to aqueous phase so as to precipitate the DNA. The DNA was pellted by centrifugation at 12000 rpm for 10 minutes. The pellet, washed with 70% ethanol was dried and dissolved in 100 µl of TE buffer (pH 7.6)

PCR was done as per the method described previously by Cascon *et al.* (1996). The different components used for the amplification of the DNA in PCR were as follows: Primers used in this study were selected on the basis of the nucleotide sequence of the 1952 bp lipase gene derived from *A. hydrophila* H3. The sequences of the two primers of 24 and 23 bases respectively, were:

Forward Primer 5'-AACCTGGTTCCG-CTCAAGCCGTTG-3'

Reverse Primer 5'-TTGCCTCGCCTC-GGCCCAGCAGCT-3'

Amplification of *lip* gene was performed with a DNA thermal cycler (DNA engine, MJ Research) with some modifications as follows: The reaction mixture consisted of 1µl of Taq polymerase

(1unit), 5 µl of 10X PCR amplification buffer (100 mM Tris-Hcl, 25 mM $MgCl_2$, 500mM KCl, pH-8.3), 3 µl of deoxynucleoside triphosphate (100µM), 0.5 µl of each primer (80 pmoles) and double distilled water upto a final volume of 50 µl. A total of 40 PCR cycles were run under the following conditions: Initial denaturation at 94^{0}C for 4 minutes, denaturation at 94^{0}C for 1 minute, primer annealing at 65^{0}C for 1 minute, DNA extension at 72^{0}C for 1.5 minutes and final extension at 72^{0}C for 5 minutes.

Agarose gel electrophoresis of the amplified product

To visualize the PCR product, agarose gel electrophoresis was performed as follows. The gel electrophoresis was performed in 1.2% agarose made in 0.5X TBE (Tris -5.4 g, Boric acid-2.75 g, 0.5 M EDTA pH-8.0-2 ml, Distilled water-1000 ml). Samples were mixed with loading buffer and loaded in slots and electrophoresed at 100 volts for three hour at room temperature. For staining, ethidium bromide at a final concentration of 4 mg/ml was added in the gel tank and gel was kept for 15 minutes and visualized under UV transilluminator.

Detection of Aeromonas hydrophila *by PCR*

Bacterial chromosomal DNA from all 9 *A. hydrophila* bacterial isolates was obtained along with *E. tarda* and *S. typhimurium*. The optimal concentration of 2 mM $MgCl_2$ was used in the reaction mixture for the amplification of the *lip* gene in the isolated genomic DNA. PCR products of desired size 760 bp were obtained in reaction mixture containing genomic DNA of the targeted organisms, *A. hydrophila*. No product was detected in other organisms. PCR product of 760 bp was obtained in all 9 isolates of *A. hydrophila*, but not in *E. tarda* and *S. typhimurium* (Fig.1).

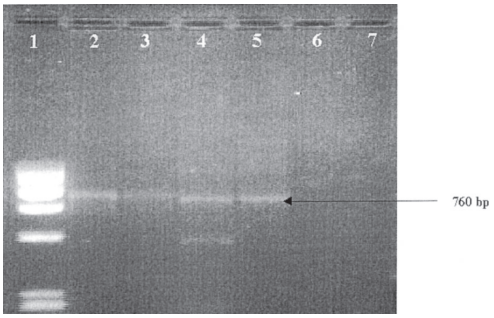

Fig. 1 Detection of *Aeromonas hydrophila* by PCR. Lane 1:Phi X 174 DNA *Hae* III digest Marker; Lane 2-5 *Aeromonas hydrophila;* Lane 6 *Salmonella typhimurium;* Lane 7 *Edwardseilla tarda*

Identification of fish pathogenic bacteria is important in the diagnosis of disease. However, conventional methods of isolation and identification are cumbersome and time consuming. The amplification of the specific DNA sequence by PCR provides a highly sensitive and specific tool for the detection of microorganism from different sources. *A. hydrophila* have also been detected by the amplification of aerolysin gene by Pollard *et al*. (1990), which targets 209 bp fragment of *aero* gene coding for the aerolysin toxin. Cascon *et al*. (1996) screened 50 strains of bacteria including 14 currently recognized DNA hybridization groups of *Aeromonas* sp., as well as other human and environmental isolates of *Aeromonas* through amplification of *lip* gene. A DNA fragment of approximately 760 bp was amplified only in the strains of *A. hydrophila*. In the present study *A. hydrophila* was detected by amplification of *lip* gene, which codes for a thermostable extra cellular lipase of *A. hydrophila*. The PCR primers designed for the specific detection of *A. hydrophila*

was tested successfully and a desired PCR product of 760 bp were obtained in reaction containing genomic DNA of *A. hydrophila*. No product was detected when genomic DNA from organisms other than *A. hydrophila* was used.

The optimal concentration of $MgCl_2$ was 2 mM in the reaction mixture. No amplification product was obtained when the concentration was 1.5 mM and non-specific amplification was obtained when the concentration was higher than 2.5 mM. The PCR conditions given by Cascon *et al.* (1996) for the amplification of the *lip* gene was modified and the annealing of the primer was done at 65^0 C. The initial denaturation of the template was done at 94^0 C for 4 mts. These modifications gave more specific results in the detection of *A. hydrophila*.

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